Comparision of Microscopic Examination and PCR for Detection of Theleriosis in Cows and Buffaloes in Urban and Peri Urban Areas of Hyderabad

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Abstract: *The study was conducted on affected cattle which were selected on the basis of clinical signs, appearance of ticks, history and presence of Theileria diagnosed by thin blood films and Polymerase Chain Reaction (PCR). Total of 300 blood samples from cows and 2400 samples from buffaloes were collected from urban and peri urban areas of Hyderabad. Results revealed 90 % and 80% prevalance of thielarial infection in cow of urban and peri-urban areas respectively. Whereas higher percentage of infection was observed in cows at urban areas as compare to peri-urban areas of Hyderabad. Similar trend was observed in bufffaloes with 65% and 75% prevalance in urban and peri-urban areas of hyderabad respectively. Overall prevlance of thielariosis in cows was higher 85% as compare to buffaloes 70% throgh microscpy method. Cows in Urabn areas showed more prevalance of theilariosis than peri-urban areas. Whereas, buffaloes showed higher percentage of prevalance in peri-urban areas as compare to urban areas of Hyderabad. Statistical analysis of the data revealed significant difference (P<0.05) between prevalance percentage between both species as well as between urban and peri-urban areas of Hyderabad. Comparative results of microscopic examination and PCR for the detection of theileriosis in cows and buffaloes revealed higher prevalence on PCR as compare to microscopic examination. This difference was observed between both species as well as between urban and peri-urban areas of Hyderabad. Overall prevalence of theileriosis was 85% and 87 % in cows and 70% and 76% in buffaloes on microscopy and PCR respectively. It is concluded that the cows were more susceptible to tick borne disease of theleiriosis than buffalo and PCR method is more reliable, specific, sensitive to detect and characterize the organism as compare to conventional method.*

I. Introduction

A large number of diseases have been incriminated to affect the production and reproduction potential of the animals. Among the diseases, tick born infections are of great importance. It has been reported that about 80% of the world cattle population is infested with ticks (Bowman *et al.,* 1996). Ticks not only cause direct losses by sucking blood of the host animal, but also transmit various blood-borne diseases e.g. Theileriosis, Babesiosis, Anaplasmosis , Trypanosomiasis etc, induce paralysis or toxicosis and cause physical damage to livestock (Durrani *et al.,* 2008 and Rajput *et al.* 2005).Protozoan diseases particularly theileriosis imposes considerable restraints on the buffaloes and cattle production. Theileriosis in buffaloes cause both population and economic losses (Durrani *et al.,* 2008). Bovine theileriosis is a tick-borne heamoprotozoan disease caused by *Theileria annulata* which is transmitted by *H.* The diagnosis of Theileriosis in acute cases is mainly based on clinical findings and microscopic examination of Giemsa's stained thin blood smears (Ismail *et al.,* 2016). Sometimes the diagnosis of *Theileria annulata* infection in buffaloes and cows, based on clinical signs is difficult, because of wide variety of disease clinical picture that may be mistaken with other diseases. Conventional method like thin blood film and lymph node smears are accepted as a method of laboratory diagnosis in cattle and buffaloes (Ramazan and Ugur, 2006). This method is not sensitive and confronts some false positive or false negative results (Riahi-Zanjani, 2014 and Shayan and Rahbari, 2005). In long standing carrier animals blood smears are negative on microscopy (Aktas *et al.*2006). The advent of the PCR coupled with the specificity of deoxyribonucleic acid (DNA) hybridization had led to the development of specific and sensitive molecular diagnostic tests to detect and characterize the organisms that cause Theileriosis (Collins *et al.*2002). The PCR method is more accurate in comparison with the serological tests such as enzyme linked immunosorbent assays (ELISA), immunoflouresent antibody test (IFAT) and indirect haemagglutination assay (IHA) as well as microscopic detection of piroplasmic forms. PCR method enabled to detect parasitic infections with clinical or without clinical signs (Almeria 2001 and Dumanli 2005 and Tavassoli *et al.,* 2013). Traditionally, detection of *Theileria* pathogens in infected animals requires the microscopic examination of stained-blood smears and serological methods. However, limitations of these methods against this parasite limit

the specificity. Molecular diagnostic assays have been developed for the detection of *Theileria* parasites, including PCR-based and reverse line blotting approaches, but these methods usually demand qualified personnel, complex instrumentation, and expensive materials. Loop-mediated isothermal amplification (LAMP) can facilitate the design of molecular assays independent of the use of sophisticated equipment (Gomes and Inácio, 2015).Therefore, the present study was designed to compare microscopic examination and PCR for detection of theleriosis in cows and buffaloes in urban and peri urban areas of Hyderabad, Pakistan.

II. Materials and Methods

The affected cattle were selected on the basis of clinical signs, appearance of ticks, history and presence of Theileria in the thin blood films and Molecular Diagnosis through Polymerase Chain Reaction (PCR). Clinically healthy (Buffaloes and cows) tick-free cattle of almost same age from 20 selected farms were used as a control. All clinically suspected animals were subjected to clinical examination of mucosal membranes (conjunctival, nasal and oral) for hemorrhages and pre-scapular lymph nodes for enlargement. Rectal temperature of each animal suffering from Bovine Theileriosis was recorded.Total of 325 blood samples from cows and 2500 samples from buffaloes were collected from tick-infested and tick-free buffaloes ,cows and calves of either sex of both species from urban and peri urban areas of Hyderabad.

Cotton swab soaked in the antiseptic 65% (alcohol) was applied for disinfection to avoid the contamination in the sample. With the help of sterile needle and syringe, 5ml of blood was taken from jugular vein and preserved on the spot in labeled tubes containing EDTA, was mixed slowly and gently to avoid hemolysis. The collection vials were soaked by rotating in between the palms for proper mixing of the anticoagulant. After blood collection, test tubes were labeled with the name of owner, patient, breed, species and identification, time and date and for the purpose of the examination. The samples were put in ice box and were brought to the central laboratory of the faculty of animal husbandry and veterinary sciences for Haematology, then after samples were sent to the molecular laboratory, department of Veterinary Parasitology, Sindh Agriculture University Tandojam. The whole-blood samples were stored at −20°C until extraction of DNA.

Microscopic diagnosis (conventional method).
Thin blood smears were made on the microscopic glass slides on the spot and were fixed in absolute alcohol for microscopy as described by Mello *et al.* (2014). The slides were held by the edges to avoid the finger prints and contamination of slide area, where the blood smear was to be made. A tiny drop of blood was placed on one end of the microscopic slide; with the edge of another slide (called as spreader slide) the drop of blood was spread across the slide at 45° angle. The smear on the slide was dried by waving the slide in air. In order to avoid any shrinkage or rupturing of the erythrocytes, the slides were immediately fixed.

Fixation of blood smears
After drying the blood smears, it was necessary to fix the films before applying a stain, so as to prevent the water dissolving the hemoglobin out of the red blood cell (RBC). The slides were fixed in 100% methyl alcohol for 1-2 minutes.

Staining of blood smears
Giemsa stain, which is one of the Romanowisky series of stains, was used for the staining of blood smears.

Stain preparation
Two grams of the Giemsa powder was added to 250 ml of glycerin in flat-bottomed flask (Borosil). Collected mixture was placed at 60^oCwater bath (Model LWB-111D, Labtech, Koria) for 2 hours. After cooling the mixture, 250 ml methyl alcohol was added and stirred on a magnetic stirrer (Model LMS-1003, Labtech, Koria) for 24 hours at room temperature. Prepared stain was poured in a dry stoppered brown bottle and stored. The filtration was done prior to stain.

Staining
The staining of the slides was done in 10% solution of Giemsa stain for five minutes and then slides were rinsed with distilled water.

Examination of Blood Smears
Slides were examined at 100X (oil immersion) objectives in a binocular electric microscope in the laboratory of the Department of veterinary Parasitology, Faculty of Animal Husbandry and Veterinary Sciences, Sindh Agriculture University Tandojam. The identification of parasites was done with the help of keys as described by Soulsby (1982)

Molecular diagnosis
Before starting the molecular diagnosis, the required material was sterilized by autoclave and pippets were disinfected with 70% alcohol.

DNA extraction
DNA from blood samples was extracted using commercial kit (TIANamp Genomic DNA Kit Cat. #: DP304) as per manufacturer's instructions.
Before start of DNA Extraction protocol, the Buffer GD and Buffer PW were prepared with appropriate volume of ethanol (100%) as indicated on the bottle and were shacked.

- The amount of 20μl of Proteinase K was mixed with 200ul frozen and anticoagulated inependorf tube and mixed thoroughly by vortexing.
- GB buffer 200ul was added in the sample and again mixed thoroughly by vortex, and incubated at $70^{o}C$ for 10 minutes to yield a homogenous solution than microcentrifuge tube to remove the drops from the inside of the lid.
- Ethanol (100%) 200 ul was added to the sample and mixed thoroughly by vortex for 15 seconds. Then later tube was briefly centrifuged to remove drops from the inside of the lid.
- The mixture was pipeted into the spin column CB3 (Provided in Kit) which placed in collection tube and centrifuged at 12000 rpm for 30 seconds.
- Flow was discarded and spin column was placed into the collection tube.
- Buffer GD 500 ul was added to spin column CB3 and centrifuged at 12000 rpm for 30 seconds and then flow was discarded and spin column was placed into the collection tube.
- Buffer PW 600 ul was added to spin column CB3 and centrifuged at 12000 rpm for 30 seconds. Again flow was discarded and spin column was placed into the collection tube.
- Similarly 600 ul buffer PW was added to spin column CB3 and centrifuged at 12000 rpm for 30 seconds. Flow was discarded and spin column was placed into the collection tube and centrifuged at 12000 rpm for 2 min to dry the membrane completely.
- The spin column CB 3 in a new clean 1.5 ml micro centrifuge tube was placed and pipeted 100 ul buffer TE was directly centered of the membrane, then incubated at room temperature (20-25 c) for 5 min and then centrifuged for 2 minutes at 12000 rpm.
- The purification column was discarded and stored 1.5 ml tube which contained DNA.
- Finally, nucleic acid concentration was measured by spectrophotometer (Nanodrop 1000, Thermo Scientific, USA).

Preparation of reagents
Preparation of TE buffer
The 121.4g of Tris (121.4 Mw) was dissolved in 1000 ml of water and solution was placed on the hot plate stirrer for mixing. The pH was maintained upto7.5 with using hydrochloric acid (HCL).
Then 372.2ml EDTA was added in 1000ml water, placed on the hot plate stirrer for mixing and the pH was adjusted up to 8 when required by using sodium hydroxide (NAOH).
For preparation of 500ml TE buffer, 5ml of Tris, 1ml of EDTA and 494ml water was mixed; pH was adjusted at 7.5 and was used for primer dilution.

Primers
Primers were purchased from Gene link USA through Worldwide Scientific Lahore, which were supplied in desalted and lyopholized form. These were reconstituted in TE buffer (10mM Tris, 1mM EDTA, pH 7.5).

The stock solution of 100pmols/ μl (100μM)
Gene link provide the exact amount of each primers supplied on the tube. The amount of forward primers was 38.7 nanomoles and reverse primer was 45.2 nanomoles. The number of nanomoles was multiplied with 10 to get the volume of TE to be added in each tube. The TE was added to the primers and then vortexed for mixing. Primer solutions were 10-fold diluted to prepare a working solution of 10 Picomoles/ μl (10μM) concentration.

DNA amplification
Theleriain buffaloes was detected by using PCR (Polymerase Chain Reaction). The PCR was performed on the extracted DNA by using primers for theileria which was designed at Mather lab., University of Rhode Island, USA (Table. 1)

Table 1 Primers used for the identification of *T. annulatta*

Primer	Sequence	Position	Characteristic
30 kDa gene N516	GTAACCTTAAAACGT	234-250	*T. annulata* specific
N517	GTTACGAACATGGGTTT	954-938	*T. annulata* specific

The PCR assay was performed in a 25µl reaction volume containing12.5µlof GoTaq Green Master Mix, 2X (Promega USA), 2.5µl of each primer, 3.5 µl Nuclease Free Water and 4 µl of DNA extracted from blood of suspected theleria-infected cattles. PCR reaction was performed in thermal cycler (Applied Biosystem 2720) and cycling conditions were as an initial denaturation for 5 minutes at 94°C followed by 40 cycles with denaturation for 45 seconds at 94°C, annealing for 45 seconds at 58°C and extension for 45 seconds at 72°C and a final extension for 7 minutes at 72°C.

Electrophoresis
Agarose gel preparation
The 2% of Agarose Gel was prepared by mixing the 1gm of agarose powder in 50ml of 1X TAE buffer in conical flask (250ml). The mixture was boiled in microwave oven for 50 seconds at full power level. After heating the flask was removed from microwave oven and allowed to cool down to 60°C and 3µl of Ethidium bromide solution (10mg/ml) was mixed in dissolved agarose gel and then allowed to cool further. The dissolved gel was placed in gel casting tray and left it till the gel was completely set (approximately 40 minutes). The 450ml 1X TAE solution was poured in electrophoresis tray. When the gel is completely formed then it was fixed in the electrophoresis tray and comb was removed.

Sample loading and running
1. The 6µl of 100bp DNA ladder (Cat. No. SM0241, Fermentas EU) was loaded in the first well of the gel
2. The 6µl of each sample (PCR product) was loaded in the well besides the well in which DNA ladder was loaded
3. Lid of Gel Electrophoresis Unit was closed and machine was set on 80 volts, for 45 minutes. However, regular visual inspection was done for to observe the progress of samples.

Gel documentation
After 45 minutes, the gel was removed from gel tray and placed in Gel Documentation System, Cleaver Scientific, Ltd, UK. The door was closed, and software operated system was used to read the bands and documented though picture. This system analysis the nucleic bands according to their molecular weight against the established DNA bands that are run in marker.

Data analysis
The Data analysis was conducted for mean prevalence at 95% confidence interval and chi square to determine the significance at 0.05 levels in groups by using the Graphpad Instat Soft-ware.

III.   Results
The study was performed in order to determine the prevalence of *theileria* species in buffaloes and cows of peri-urban and urban areas of Hyderabad, Sindh, Pakistan. The prevalence of Theleriosis was determined by conventional as well as molecular methods in naturally tick-infested cows and buffaloes. A total of 2825 animals were registered in the study area. Total of 2500 samples from buffaloes and 325 cows were collected for detection of *theileria* spp during 2013-2014.

Prevalence of theilerial infection in naturally tick infested cow and buffaloes
Prevalence of Theiarial infection was determined by microscopic examination of blood as well as through polymerase chain reaction (PCR) using specific primers. For microscopic detection of Theilerail infection, thin blood smears were examined under low magnification at 40X. Presence of *Thelieria* was confirmed on the basis of physical characteristics of theileria Species i.e.,   round, oval, elongate, ring and anaplasmoid shapes structure (Fig-7). Blood smears of naturally tick-infested cows and buffaloes with typical physical characteristics of *Theileria Spp* were considered as positive for infection.

Prevalence of theleriosis in cows and buffaloes on the basis of microscopic examination:
Blood smears of total three hundered (n=300) cows and twenty four hundred (n=2400) buffaloes from urban and preiurban area of Hyderabad that were suspected for thilerial infection were examined. Results revealed 90 % and 80% prevalance of thielarial infection in cow of urban and peri-urban areas respectively. Whereas higher percentage of infection was observed in peri-urban areas as compare to urban areas of

Hyderabad. Vice versa trend was observed in bufffaloes with 65% and 75% prevalnce in urban and peri-urban areas respectively. Overall prevlance of thielariosis in cows was higher 85% than buffalo which is 70%. Cows and bufflaoes in Urabn areas showed less prevalance of theilariosis than peri-urban areas. Statistical analysis of the data revealed significant difference (P<0.05) between prevalance percentage   between both species as well as between urban and perii-urban areas of Hyderabad (table 2).

Prevalence of theilariosis in cows and buffaloes on the basis of polymerase chainreaction (PCR)

Prevalence of theleriasis in cow and buffaloes as detected by PCR was higher in cows 91% and 83% in urban and peri-urban areas of Hyderabad. Prevalence of theileriosis in cows of urban areas was higher 91% than that of pri-urban areas 83%. Whereas, less number of buffaloes were found positive for Theilariosis on PCR in urban areas 68% as compare to peri-urban areas 85%. Cows were found more susceptible to tick infestation than buffaloes as recorded by PCR results. Overall infection rate in cows was calculated as 85% that is higher than that in buffaloes 76%. Statistical analysis of the data revealed significant difference (P<0.01) between prevalance percentage between both species as well as between urban and peri-urban areas of Hyyderabad (table 3).

Comparision of microscopic examination and PCR for detection of theleriosis in cows   and buffaloes in urban and peri urban areas of Hyderabad

Comparative results of microscopic examination and PCR for the detection of theileriosis in cows and buffaloes revealed higher prevalence on PCR as compare to microscopic examination. This difference was observed between both species as well as between different areas. Overall prevalence of theileriosis in cows was 85% and 87 % on microscopy and PCR respectively. Same trend appeared in buffaloes where higher prevalence was recorded on PCR in both urban and peri-urban areas of Hyderabad (Fig-2).

IV.   Discussion

The study was conducted to evaluate the prevalence of theileriosis in cows and buffaloes through conventional and molecular detection method in urban and peri urban areas of Hyderabad. Variation in occurrence and severity of disease is mostly depending upon tick population in the areas as well as climatic conditions that provides favour to tick infestation in cows and buffaloes. In this study, the cases of the disease were relatively more prevalent (85%) in buffaloes reared in peri-urban farms as compare to 68% in urban areas of Hyderabad. The variation in infection rates of domestic ruminants with tick-borne pathogens is related to several factors including the presence and abundance of tick species which act as vectors for specific pathogens, genetic variation among animals and breeds in resistance, and the presence of wild-life reservoirs (Chaisi *et al.,* 2013). Gender and age were not found to be significantly associated with positivity for Theileria infection in the surveyed animals. This is in agreement with other studies on *Theileria spp.* infections in small ruminants and cattle (Flach and Ouhelli, 1992; Razmi *et al.,* 2006 and Perera *et al.,* 2015).

Development of the more advance molecular biology techniques such as PCR have made it possible to diagnosis disease at sub-clinical level with high sensitivity and specificity which otherwise impossible with conventional methods of diagnosis. In present study we compare the conventional method i.e., semear staining with molecular techniques (PCR) to determine Theileria infection in cows and buffaloes at peri-urban and urban areas of Hyderabad. We found that PCR was much more sensitive than staining method. Overall prevalence of Theileriosis in cows was 85% and 87% on microscopy and PCR respectively. Similar findings have been reported by Lalchandani (2001) who observed higher prevalence percentage of theileriosis in Kundhi buffaloes on PCR. Oliveira *et al.,* (1995) also found that PCR is highly sensitive test for detection of *Theileria annulata.* They recorded (75%) positive theileriosis compared to 22% positive on blood smear examination.

In conclusion, the geographical location of Pakistan is in the warm climate zones (WCZS) of the world, along with large scaled cross-breeding programs has made it an endemic area for Theileriosis. Theileriosis has profound effects on hematological and milk values and is responsible for mortality and losses in production with its highest incidence during spring to late summer. The future needs include latest diagnostic techniques like PCR, appropriate tick eradication programs, controlled crossbreeding, and thoroughly monitored preventive medicine programs in order to reduce both the incidence and prevalence of the disease.

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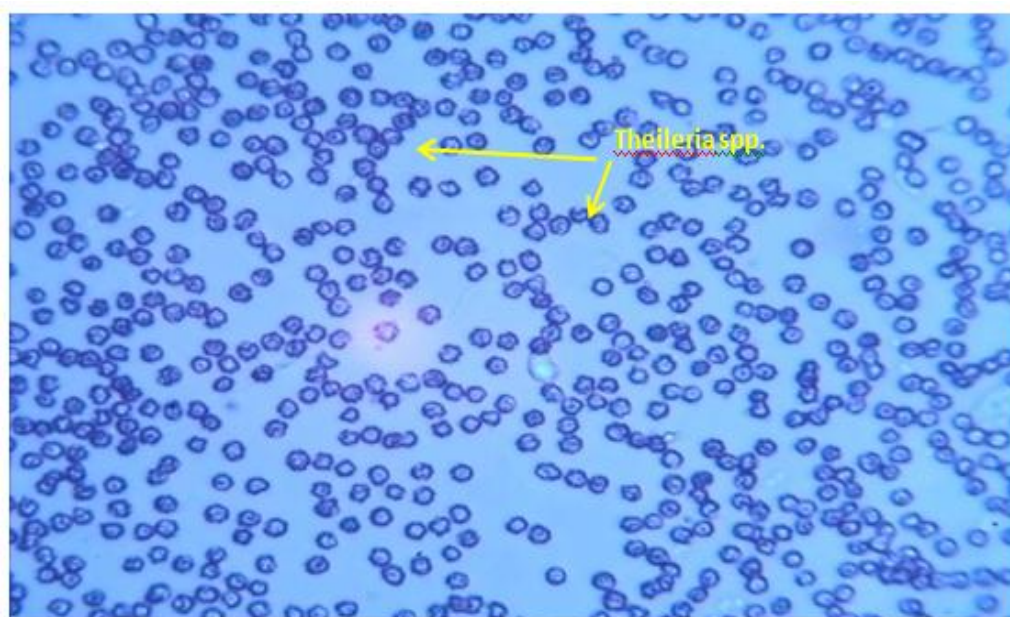
Plate-1 Microscopic examination of thin blood smear of buffalo at 40X for diagnosis of Theilaria spp.
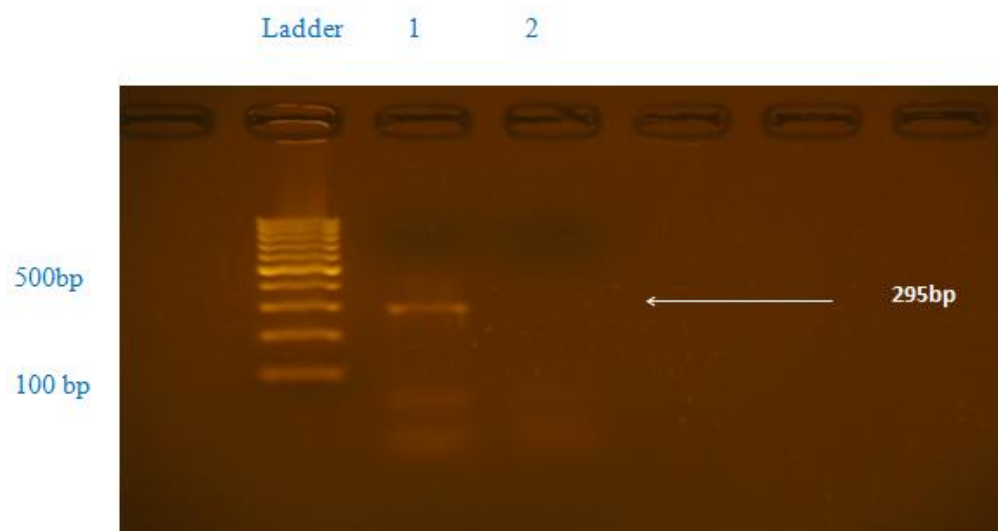
Fig-2 Gel electrophoresis of amplified PCR product of *Theileria annulata* of cow and buffalo blood DNA.

Table 2 Number of theleriosis cases in cows and buffaloes diagnosed through the microscopy in urban and peri-urban areas of Hyderabad.

Sr. No	Areas Screened	Cows		Buffaloes		Percentage
		No Examined	No Positive	No examined	No Positive (%age)	
01	Urban	150	135	1200	779	**48.12**
02	Peri-urban	150	120	1200	901	**51.88**
Overall		**300 (6.47)**	**255 (5.5%)**	**2400 (51.10)**	**1835 (38.31)**	**100**

Chi-square for trend= 4.659 (1 degree of freedom)
The positive cases from examined cows were significantly higher in linear trend (P<0.05) than positive cases of buffaloes in urban, peri-urban areas and overall in Hyderabad.

Table-3 Number of theleriosis cases in cows and buffaloes confirmed through the polymerase chain reaction (PCR) in urban and peri-urban areas of Hyderabad.

Sr. No	Areas Screened	Cows		Buffaloes		Percentage
		No Examined	No Positive	No examined	No Positive (%age)	
01	Urban	150	112	1200	820	48.09%
02	Peri-urban	150	125	1200	1015	51.91%
Overall		300 6.25%	262 54.6%	2400 50.03%	1835 38.25%	100%

Chi-square for trend= 9.324 (1 degree of freedom)
The positive cases from examined cows were significantly higher in linear trend (P<0.01) than positive cases of buffaloes in urban, peri-urban areas and overall in Hyderabad.

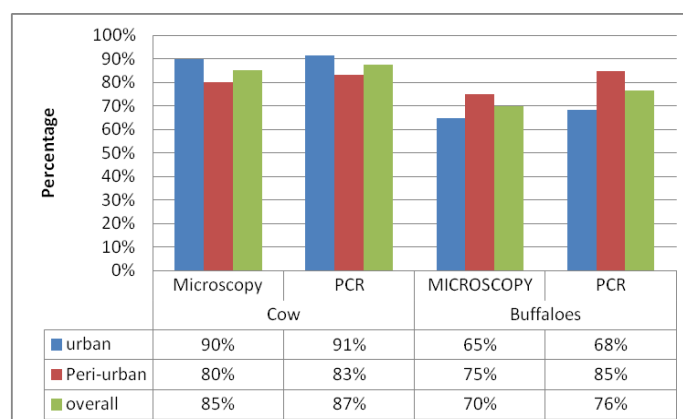|	Microscopy (Cow)	PCR (Cow)	MICROSCOPY (Buffaloes)	PCR (Buffaloes)
urban	90%	91%	65%	68%
Peri-urban	80%	83%	75%	85%
overall	85%	87%	70%	76%

Fig-2 Prevalance of thelerioasis in cow and buffaloes on the basis of microcscopy and PCR in urban, peri-urban areas and ovarall in Hyderabad.